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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,380,970**, on April 8, 2002, by **SARISSA INC.**, assignee of James D. Koropatnick and Mark D. Vincent for "Combination Antisense Oligonucleotides for Use in Cancer Therapies".

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ANTISENSE OLIGONUCLEOTIDE STRATEGIES FOR THE ENHANCEMENT OF CANCER THERAPIES

ABSTRACT

The present invention provides for effective combinations of antisense oligonucleotides directed against thymidylate synthase mRNA for use in cancer therapies. Combinations of antisense oligonucleotides have enhance activity compared to the activity of the individual antisense oligonucleotides when used alone or in conjunction with known and novel chemotherapeutic agents. Such antisense oligonucleotide combinations constitute improved antisense therapies with application to a variety of cancers or proliferative disorders, including drug resistant cancers.

FIELD OF THE INVENTION

The present invention pertains to the field of antisense oligonucleotides in cancer therapies.

BACKGROUND

Antisense oligodeoxynucleotides (ODNs) are synthetic nucleic acid molecules designed to hybridize by Watson-Crick base-pairing with mRNA sequences and target them for degradation by ribonuclease H. Several antisense ODNs targeting a variety of molecules have antiproliferative effects against neoplastic cells *in vitro* and *in vivo* (Gewirtz, 2000), and several have demonstrated anti-tumor activity and limited toxicity in Phase I clinical trials (Smith and Wickstrom, 2000). It has, however been suggested that antisense approaches or technology is failing to live up to its promise (Gewirtz *et al.*, 1996; Gewirtz, 2000; Lebedeva and Stein, 2001), in terms of the prevalence of non-specific or non-antisense effects, and in terms of the 'limited' effects of antisense ODNs as single agents in various settings, especially human clinical trials (Gewirtz, 2000; Waters *et al.*, 2000). Furthermore the question of the effective delivery and penetration of antisense ODNs to tumor sites is also cited as a deficiency in current antisense therapy protocols.

A handful of studies have examined the use of multiple antisense ODNs and these ODNs were designed to distinct mRNA targets. There are reports in which multiple antisense ODNs exhibit cooperative or more than additive effects (Normanno *et al.*, 1996; Skorski *et al.*,1995; Skorski *et al.*, 1996). In the first, antisense ODNs targeting 3 members of the epidermal growth factor family (cripto, amphiregulin, and transforming growth factor α) were used in combinations against human colon carcinoma cells *in vitro*. The combination of all 3 ODNs was particulary effective at inhibiting the anchorage-independent growth of the tumor cell line (Normanno *et al.*, 1996).

In addition, a combination therapy using antisense ODNs targeting BCR-ABL and c-myc was more effective than either treatment alone at delaying tumor growth and prolonging survival in a chronic myelogenous leukemia model *in vitro* and *in vivo* (Skorski *et al.*, 1995; Skorski *et al.*, 1996). BCR-ABL and c-*myc*, had both been targeted independently with antisense ODNs, resulting in reduced tumor growth in a variety of *in vitro* and *in vivo* tumor models

(Leonetti *et al.*, 1996; Skorski *et al.*, 1994; Szczylik *et al.*, 1991). In addition, antisense ODNs to c-*myc* enhances cytotoxicity of a number of chemotherapeutic drugs in combination treatments (Leonetti *et al.*, 1999; Mizutani *et al.*, 1994). The c-*myc* protein is a downstream effector of BCR-ABL, and is required for transformation of hematopoetic cells by BCR-ABL (Sawyers *et al.*, 1992). When the antisense ODNs against these two targets were combined, specific decreases in both target mRNAs correlated with decreased hematopoeitic colony-forming ability *in vitro* and substantially prolonged the survival of tumor-bearing mice compared with either antisense ODN alone (Skorski *et al.*, 1996).

In other studies, the cooperative effects of antisense ODNs were not clearly demonstrated (Sato *et al.*, 2000; Traidej *et al.*, 2000). Furthermore, none of these reports test the efficacy of employing combinations of antisense ODNs against different regions of the same mRNA target in order to enhance the effects of antisense oligonucleotide treatment.

There are many genes which express proteins that are implicated in cancers and have been targeted by cancer therapies using chemotherapeutics. An example of such a gene is the gene encoding for thymidylate synthase (TS). TS is an essential enzyme in *de novo* production of thymidylate (Carreras and Santi, 1995) and, due to its crucial role in DNA synthesis and cell proliferation, has been an important target for cancer chemotherapy for many years (Danenberg, 1977; Danenberg *et al.*, 1999).

The TS inhibitors, such as 5-fluorouracil (5-FU) and its variants, and raltitrexed (Tomudex®) have become integral drugs in standard treatments for colorectal cancer (Papamichael, 1999). Although reasonably successful in clinical use, both of these drugs suffer from problems of dose-limiting toxicity and outgrowth of resistant cells, motivating the continued search for alternative treatments, such as antisense ODNs that target and impact upon the expression of TS mRNA (US Patent No. 6087489, WO 99/15648 and WO 98/49287). A specific antisense oligonucleotides targeting the 3' untranslated region of TS mRNA, has been shown to down-regulate the expression of TS, inhibit neoplastic cell proliferation (Berg *et al.*, 2001) and sensitize HeLa cells to raltitrexed, 5-FU and 5-fluorodeoxyuridine (5-FudR) (Ferguson, 1999).

More recently, initial observations of the effect of combinations of antisense ODNs complementary to TS mRNA on HeLa cells suggest that the use of certain combinations of antisense oligonucleotides may elicit a more than additive antiproliferative effect compared to the effect elicited by the individual antisense oligonucleotides alone. There was also a preliminary observation with respect to a specific combination of antisense ODNs that appeared to augment the cytotoxicity of a combination treatment of raltitrexed and 5-FudR on HeLa cells, as compared to the chemosensitizing effect exerted by one of the antisense oligonucleotides alone, i.e. the antisense oligonucleotide previously reported on by Berg *et al.*, 2001 and Ferguson *et al.*, 1999 (Berg *et al.*, AACR, Abstract #3903).

In the present application strategies for the development and design of efficacious combinations of antisense ODNs that target different regions of a TS is described. The strategies provide for an approach to the rational or empirical design of combination antisense ODNs effective as cancer thereapies. This represents a novel strategy to developing new cancer therapies or improving known cancer therapies with a wide range of applications.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

In accordance with an aspect of the present invention, there is provided a combination of two or more antisense oligonucleotides complementary to a thymidylate synthase mRNA for use in the treatment of cancer together with one or more chemotherapeutic agents, wherein the use of the combination enhances the anti-tumor effect of standard doses of the one or more chemotherapeutic agents.

In accordance with an aspect of the present invention, there is provided a combination of two or more antisense oligonucleotides complementary to a thymidylate synthase mRNA for use in the treatment of cancer together with one or more chemotherapeutic agents, wherein the

use of the combination reduces the amount of chemotherapeutic required to effectively treat a mammal with cancer.

In accordance with an aspect of the invention, a combination of two or more antisense oligonucleotides complementary to a thymidylate synthase mRNA for use together with one or more chemotherapeutic agents to treat a mammal.

In accordance with an aspect of the present invention, there is provided a combination of two or more antisense oligonucleotides for use in the treatment of cancer together with one or more chemotherapeutic agents to treat a mammal, wherein the sequences of two oligonucleotides of the combination are selected from the group consisting of SEQ ID NO: 1 and 2, or SEQ ID NO: 1 and 3.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Combinations of ODNs inhibit proliferation of HeLa cells more effectively than individual ODNs. HeLa cells were treated with 50 nM of each of the indicated ODNs as represented by SEQ ID NO: 1 in combination with either SEQ ID NO: 2, 3, 4, 5, or 6 (A), or with TS antisense ODNs SEQ ID NO: 1, 2, and 3 individually or in a 1:1 ratio to yield the combined doses indicated (B) and (C), and counted 4 days later. In (B) and (C), the total ODN concentration in each treatment was made up to 100 nM using the control scrambled ODN SEQ ID NO: 4. Shown are the mean proliferation (± SD) relative to cells treated with ODNs SEQ ID NOs: 1 + 2 (A), or control ODN SEQ ID NO: 4 only (B) and (C), for 3 flasks from representative experiments. Asterisks indicate significant decreases in proliferation ($P<0.05$, Student *t* test) when compared to cells treated with ODNs SEQ ID NOs: 1 + 4 (A), or with either antisense ODN alone (B) and (C). A dagger indicates a significant decrease in proliferation only when compared to cells treated with ODN SEQ ID NO: 1 alone.

Figure 2. Quantitation of TS and GAPDH mRNA levels and TS protein levels in HeLa cells treated with ODN combinations. HeLa cells were treated with the indicated ODNs (100 nM total) for 24 or 48 hours, and RT-PCR was performed and quantitated as described in Examples. (A) Ethidium bromide-stained gel shows GAPDH and TS PCR products from a representative experiment. (B) Quantitation of gel shown in (A) and others showing

reductions in TS mRNA following various ODN treatments; ODN SEQ ID NO: 3 did not reduce TS mRNA levels (data not shown). (C) HeLa cells were treated for 24 hours with 25 or 50 nM of the individual ODNs, or with 25 nM of each ODN, and TS protein levels were quantitated by 5-FdUMP binding as described in Examples.

Figure 3. Flow cytometry analysis of cell cycle profiles in HeLa cells treated with ODN combinations. HeLa cells were treated with 200 nM of control ODN SEQ ID NO: 4 (A), ODN SEQ ID NO: 1 (B), or ODN SEQ ID NO: 2 (C), or with 100 nM each of ODNs SEQ ID NO: 1 + 2 (D) for 48 hours, and collected for analysis by flow cytometry as described in Examples. Insets show the fraction of cells in G0/G1, S, and G2/M, determined using MultiCycle software.

Figure 4. Sensitivity of HeLa cells to raltitrexed (A) and 5-FUdR (B) following treatment with combinations of TS antisense ODNs. (A) HeLa cells were treated with 100 nM (total) of the indicated ODNs for 4 hours, then the indicated concentration of raltitrexed was added, and the cells were counted 4 days later. Shown are the mean proliferation (± SD) relative to cells treated with each ODN mixture in the absence of raltitrexed for 3 flasks from a representative experiment. (B) Experiment with ODN combinations and 5-FUdR.

Figure 5. Enhanced sensitivity of HeLa cells to lower doses of raltitrexed (A) and 5-FUdR (B) following treatment with lower doses of ODN SEQ ID NO: 1 at which concentrations the ODN has no antiproliferative effect. HeLa cells were treated with the indicated concentration of ODN SEQ ID NO: 1, mixed with ODN SEQ ID NO: 4 to total 50 nM, for 4 hours. The indicated concentration of raltitrexed (A) or 5-FUdR (B) was added, and the cells were counted 4 days later. Shown are the mean proliferation (± SD) relative to cells treated with each concentration of ODN SEQ ID NO: 1 in the absence of drug for 3 flasks from a representative experiment. The cells were highly sensitized to TS-targeting chemotherapeutic drugs.

Figure 6. Lower doses of ODN combinations hyper-sensitize HeLa cells to 5-FUdR, compared to ODN SEQ ID NO: 1 alone.

Figure 7. Schematic representation of ODN structures.

DETAILED DESCRIPTION OF THE INVENTION

The invention generally provides for combinations of antisense ODNs directed against a TS mRNA that can be used in combination with traditional and novel chemotherapeutic drugs in cancer therapies. In one embodiment the combinations of antisense ODNs are used in conjunction with a chemotherapeutic reduce the number of neoplastic cells in a patient compared to the use of the individual ODNs with the chemotherapeutic. In another embodiment the antisense ODNs enhance the effect of a chemotherapeutic against drug resistant cancers. In yet another embodiment the antisense ODN combinations of the invention enhance the effect of a chemotherapeutic against drug sensitive cancer. In still yet another embodiment the combinations of antisense oligonucleotides of the invention are used to reduce the amount of chemotherapeutic necessary to elicit an effective anti-cancer response. In a specific embodiment selected antisense ODN combinations used in conjunction with a chemotherapeutic agent reduce the number of neoplastic cells in a patient to a greater extent than the individual ODNs with the chemotherapeutic agent. In another specific embodiment selected antisense ODN combinations used in conjunction with a chemotherapeutic agent reduce the number of neoplastic cells in a patient to a greater extent than the chemotherapeutic alone. In still yet another specific embodiment, selected antisense ODN combinations are used alone to reduce the number of neoplastic cells in a patient.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complementary to the mRNA of a thymidylate synthase gene, the 'target'. In the context of the present invention, the target gene has been implicated in processes that can lead to the development of cancer in a mammal and drug resistant cancers and is a suitable target for cancer therapies.

The term "selectively hybridize" as used herein refers to the ability of a nucleic acid to bind detectably and specifically to a second nucleic acid. Polynucleotides, oligonucleotides and fragments thereof selectively hybridize to target nucleic acid strands under hybridization and wash conditions that minimize appreciable amounts of detectable binding to nonspecific nucleic acids. High stringency conditions can be used to achieve selective hybridization conditions as known in the art and discussed herein. Typically, hybridization and washing conditions are performed at high stringency according to conventional hybridization procedures. Washing conditions are typically 1-3 x SSC, 0.1-1% SDS, 50-70°C with a change of wash solution after about 5-30 minutes.

The term "corresponds to" as used herein with reference to nucleic acid sequences means a polynucleotide sequence that is identical to all or a portion of a reference polynucleotide sequence. In contradistinction, the term "complementary to" is used herein to mean that the polynucleotide sequence is identical to all or a portion of the complement of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The following terms are used herein to describe the sequence relationships between two or more polynucleotides: "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity." A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length mRNA or mRNA sequence, or may comprise a complete mRNA or mRNA sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (*i.e.* a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a

reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.* gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2:482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* (U.S.A.) 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 573 Science Dr., Madison, WI), or by inspection, and the best alignment (*i.e.* resulting in the highest percentage of identity over the comparison window) generated by the various methods is selected.

The term "sequence identity" means that two polynucleotide sequences are identical (*i.e.* on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.* A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.* the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 30 percent sequence identity, often at least 50 percent sequence identity, and more usually at least 60 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, and frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

Antisense Molecules of the Present Invention

Selection and characteristics

"Targeting" an antisense compound to a thymidylate synthase mRNA, in the context of the present invention, is a multistep process. The process usually begins with the identification of a target nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, in one embodiment the target is the gene encoding TS. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, *e.g.* detection or modulation of expression of the protein encoded by the gene, will result. Once the target site or sites have been identified, oligonucleotides are chosen that are sufficiently complementary (*i.e.* hybridize with sufficient strength and specificity) to the target mRNA to give the desired result.

Candidate antisense ODNs are synthesized according to methods known in the art and sequences selected so as to be complementary to TS mRNA and so as to minimize non-specific interactions with other non-TS nucleic acids. ODNs are then further screened according to the methods described in the Examples for measuring the effect of ODNs on the amount of TS mRNA and TS protein in neoplastic cells. Suitable ODNs for the combinations of the invention are those ODNs which do not increase the amount of TS mRNA and/or TS protein in neoplastic cells. In an embodiment of the invention, at least one antisense ODN of a combination of the invention reduces the amounts of TS mRNA and TS protein in neoplastic cells.

Generally, there are five regions of a mRNA that may be targeted for antisense modulation: the 5' untranslated region (5'-UTR), the translation initiation or start codon region, the open reading frame (ORF), the translation termination or stop codon region and the 3' untranslated region (3'-UTR). Regions of a mRNA may be targeted, wherein known regulatory sequence elements have been identified (e.g. for post-transciptional control and mRNA stability), or that are unique, for example, to a group of mRNAs encoding for similar proteins.

In an embodiment of the invention, antisense ODNs target the 3'-UTR region of a TS mRNA. In another embodiment of the invention, antisense ODNs in combination target the 3'-UTR

region and coding region of a TS mRNA. In another embodiment of the invention, antisense ODNs in combination target the 3'UTR and 5'UTR of the TS mRNA. In yet another embodiment of the invention, antisense ODNs in combination target the 3'UTR and region spanning the stop codon of the TS mRNA. In yet another embodiment of the invention, antisense ODNs in combination target the 3'UTR and region spanning the start codon of the TS mRNA. In yet a further embodiment of the invention, antisense ODNs in combination target the regions spanning the start and stop codon of the TS mRNA. In yet a further embodiment of the invention, antisense ODNs in combination target the 5'UTR and regions spanning the stop codon of the TS mRNA. In still another embodiment of the invention, antisense ODNs in combination target the 5'UTR region of the TS mRNA. In still another embodiment of the invention, antisense ODNs in combination target the 5'UTR and coding regions of the TS mRNA.

The terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding preprotachykinin regardless of the sequence(s) of such codons.

As is known in the art, some eukaryotic transcripts are directly translated, however, most mammalian ORFs contain one or more sequences, known as "introns," which are excised from a transcript before it is translated; the expressed (unexcised) portions of the ORF are referred to as "exons" (Alberts *et al.*, (1983) *Molecular Biology of the Cell*, Garland Publishing Inc., New York, pp. 411-415). In the context of the present invention, both introns and exons may serve as targets for antisense.

In some instances, an ORF may also contain one or more sites that may be targeted for antisense modulation due to some functional significance *in vivo*. Examples of the latter types of sites include intragenic stem-loop structures (see, for example, U.S. Pat. No. 5,512,438)

and, in unprocessed mRNA molecules, intron/exon splice sites. In addition, mRNA molecules possess a 5′ cap region that may also serve as a target for antisense. The 5' cap of a mRNA comprises an N^7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of a mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap.

There are also elements in the 3'UTR region which can impact upon message stability, including examples of unique *cis*-elements that interact with trans-acting proteins to control mRNA turnover rates (Hake and Richer, (1997) *Biochim. Biophys. Acta* 1332: M31-M38). In addition, the polyadenylated tail can serve several functions impacting upon translation efficiency and message turnover, for example, by protecting the message from degradation, depending on the length of the poly 'A' tail (Ford *et al*, (1997) *Mol. Cell. Biol.* 17:398-406).

Thus, the antisense oligonucleotides according to the present invention can be complementary to regions of the complete target gene including the introns, or the antisense oligonucleotides can be complementary to part of the mRNA region from the target gene.

The antisense oligonucleotides in accordance with the present invention are selected from a sequence complementary to the target gene such that the sequence exhibits the least likelihood of forming duplexes, hair-pins, or of containing homooligomer / sequence repeats. The oligonucleotide may further contain a GC clamp. One skilled in the art will appreciate that these properties can be determined qualitatively using various computer modelling programs, for example, the program OLIGO® Primer Analysis Software, Version 5.0 (distributed by National Biosciences, Inc., Plymouth, MN).

Alternatively, the antisense oligonucleotides can be selected on the basis that the sequence is highly conserved for the target gene between two or more species. These properties can be determined using the BLASTN program (Altschul, *et al.*, (1990) *J. Mol. Biol.*, 215:403-10) of the University of Wisconsin Computer group (GCG) software (Devereux. *et al.*, (1984) *Nucleic Acids Res.*, 12:387-395) with the National Center for Biotechnology Information (NCBI) databases.

In order to be effective, antisense oligonucleotides are typically between 7 and 100 nucleotides in length. In one embodiment of the present invention the antisense oligonucleotides comprise from at least about 7 to about 50 nucleotides, or nucleotide analogues. In a related embodiment the antisense oligonucleotides comprise from about 12 to about 35 nucleotides, or nucleotide analogues, and in another embodiment from about 15 to about 25 nucleotides, or nucleotide analogues.

It is understood in the art that an antisense oligonucleotide need not have 100% identity with the complement of its target sequence. The antisense oligonucleotides in accordance with the present invention have a sequence that is at least about 75% identical to the complement of target sequence. In one embodiment of the present invention, the antisense oligonucleotides have a sequence that is at least about 90% identical to the complement of the target sequence. In a related embodiment, they have a sequence that is at least about 95% identical to the complement of target sequence, allowing for gaps or mismatches of several bases. Identity can be determined, for example, by using the BLASTN program of the University of Wisconsin Computer Group (GCG) software.

In order for the antisense oligonucleotides of the present invention to function in inhibiting expression of the target mRNA, it is necessary that they demonstrate adequate specificity for the target sequence and do not bind to other sequences in the cell. Therefore, in addition to possessing an appropriate level of sequence identity to the complement of the target sequence, the antisense oligonucleotides of the present invention should not closely resemble other known sequences. The antisense oligonucleotides of the present invention, therefore, preferably have have less than 15 nucleotides identical to any other sequence, more preferably less than 12 nucleotides identical and most preferably less than 7 nucleotides identical to any other sequence.

It will, however, be appreciated by one skilled in the art that the degree of acceptable identity between sequences may vary, for example, according to the length of the antisense oligonucleotides and the relative position of the identical nucleotides in the sequences within a comparison window, such that greater than a 15 nucleotide identity may exist, and the antisense ODN still demonstrates adequate specificity for a target sequence. The identity of the antisense oligonucleotides of the present invention to other sequences can be determined,

for example, through the use of the BLASTN program and the NCBI databases as indicated above.

Modifications to Antisense Oligonucleotides

The term "antisense oligonucleotides" as used herein includes other oligomeric antisense compounds, including oligonucleotide mimetics, modified oligonucleotides, and chimeric antisense compounds. Chimeric antisense compounds are antisense compounds that contain two or more chemically distinct regions, each made up of at least one monomer unit.

Thus, in the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), or RNA or DNA mimetics. This term, therefore, includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake or tissue penetration, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. In the context of the present invention, an antisense oligonucleotide is "nuclease resistant" when it has either been modified such that it is not susceptible to degradation by DNA and RNA nucleases, or alternatively, has been placed in a delivery vehicle which in itself protects the oligonucleotide from DNA or RNA nucleases. Nuclease resistant oligonucleotides include, for example, methyl phosphonates, phosphorothioates, phosphorodithioates, phosphotriesters, and morpholino oligomers. Suitable delivery vehicles for conferring nuclease resistance include, for example, liposomes.

As is known in the art, a nucleoside is a base-sugar combination and a nucleotide is a nucleoside that further includes a phosphate group covalently linked to the sugar portion of the nucleoside. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound, with the normal linkage or backbone of RNA and DNA being a 3' to 5' phosphodiester linkage. Specific examples of antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include both those that retain a phosphorus atom

in the backbone and those that lack a phosphorus atom in the backbone. For the purposes of the present invention, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Exemplary modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Exemplary modified oligonucleotide backbones that do not include a phosphorus atom are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. Such backbones include morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulphone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulphamate backbones; methyleneimino and methylenehydrazino backbones; sulphonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

The present invention also contemplates oligonucleotide mimetics in which both the sugar and the internucleoside linkage of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. An example of such an oligonucleotide mimetic, which has been shown to have excellent hybridization properties, is a peptide nucleic acid (PNA) [Nielsen *et al.*, *Science*, **254**:1497-1500 (1991)]. In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases

are retained and are bound directly or indirectly to aza-nitrogen atoms of the amide portion of the backbone.

Modified oligonucleotides may also contain one or more substituted sugar moieties. For example, oligonucleotides may comprise sugars with one of the following substituents at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Examples of such groups are: $O[(CH_2)_n O]_m CH_3$, $O(CH_2)_n OCH_3$, $O(CH_2)_n NH_2$, $O(CH_2)_n CH_3$, $O(CH_2)_n ONH_2$, and $O(CH_2)_n ON[(CH_2)_n CH_3)]_2$, where n and m are from 1 to about 10. Alternatively, the oligonucleotides may comprise one of the following substituents at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, $SO_2 CH_3$, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Specific examples include 2'-methoxyethoxy (2'-O--$CH_2 CH_2 OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) [Martin *et al.*, *Helv. Chim. Acta*, 78:486-504(1995)], 2'-dimethylaminooxyethoxy ($O(CH_2)_2 ON(CH_3)_2$ group, also known as 2'-DMAOE), 2'-methoxy (2'-O--CH_3), 2'-aminopropoxy (2'-$OCH_2 CH_2 CH_2 NH_2$) and 2'-fluoro (2'-F). In a specific embodiment of the invention, the antisense ODNs are methoxy-ethoxy gapped as described in the Examples.

Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Oligonucleotides may also include modifications or substitutions to the nucleobase. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5- hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other

alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808; The Concise Encyclopedia Of Polymer Science And Engineering, (1990) pp 858-859, Kroschwitz, J. I., ed. John Wiley & Sons; Englisch *et al.*, *Angewandte Chemie, Int. Ed.*, 30:613 (1991); and Sanghvi, Y. S., (1993) *Antisense Research and Applications*, pp 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C [Sanghvi, Y. S., (1993) *Antisense Research and Applications*, pp 276-278, Crooke, S. T. and Lebleu, B., ed., CRC Press, Boca Raton].

Another oligonucleotide modification included in the present invention is the chemically linkage to the oligonucleotide of one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include, but are not limited to, lipid moieties such as a cholesterol moiety [Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:6553-6556 (1989)], cholic acid [Manoharan *et al.*, *Bioorg. Med. Chem. Let.*, 4:1053-1060 (1994)], a thioether, *e.g.* hexyl-S-tritylthiol [Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, 660:306-309 (1992); Manoharan *et al.*, *Bioorg. Med. Chem. Lett.*, 3:2765-2770 (1993)], a thiocholesterol [Oberhauser *et al.*, *Nucl. Acids Res.*, 20:533-538 (1992)], an aliphatic chain, *e.g.* dodecandiol or undecyl residues [Saison-Behmoaras *et al.*, *EMBO J.*, 10:1111-1118 (1991); Kabanov *et al.*, *FEBS Lett.*, 259:327-330 (1990); Svinarchuk *et al.*, *Biochimie*, 75:49-54 (1993)], a phospholipid, *e.g.* di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate [Manoharan *et al.*, *Tetrahedron Lett.*, 36:3651-3654 (1995); Shea *et al.*, *Nucl. Acids Res.*, 18:3777-3783 (1990)], a polyamine or a polyethylene glycol chain [Manoharan *et al.*, *Nucleosides & Nucleotides*,

14:969-973 (1995)], or adamantane acetic acid [Manoharan *et al.*, *Tetrahedron Lett.*, 36:3651-3654 (1995)], a palmityl moiety [Mishra *et al.*, *Biochim. Biophys. Acta*, 1264:229-237 (1995)], or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety [Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, 277:923-937 (1996)].

As used herein and unless specifically defined otherwise, "alkyl" refers to monovalent alkyl groups having from 1 to 20 carbon atoms and more preferably, 1 to 6 carbon atoms, for example, methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl and n-hexyl . The term "aryl" may refer to an unsaturated aromatic carbon ring from 6 to 14 carbon atoms (e.g. phenyl), multiple fused rings (e.g. napthyl). The term cycloalkyl refers to cyclic alkyl groups from 3 to 20 carbon atoms and may include by way of example, cyclo -propyl, cyclo -butyl, cyclo - pentyl, cyclo -octyl or adamantanyl (multiple condensed ring structure).

One skilled in the art will recognize that it is not necessary for all positions in a given oligonucleotide to be uniformly modified. The present invention, therefore, contemplates the incorporation of more than one of the aforementioned modifications into a single oligonucleotide or even at a single nucleoside within the oligonucleotide. The present invention further includes antisense compounds that are chimeric compounds. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

The present invention further contemplates antisense oligonucleotides that contain groups for improving the pharmacokinetic properties of the oligonucleotide, or groups for improving the pharmacodynamic properties of the oligonucleotide.

Preparation of the Antisense Oligonucleotides

The antisense oligonucleotides of the present invention can be prepared by conventional techniques well-known to those skilled in the art (US Patent No. 6087489). For example, the oligonucleotides can be prepared using solid-phase synthesis using commercially available equipment, such as the equipment available from Applied Biosystems Canada Inc., Mississauga, Canada. As is well-known in the art, modified oligonucleotides, such as phosphorothioates and alkylated derivatives, can also be readily prepared by similar methods.

The isolation and purification of antisense oligonucleotides can be accomplished using, for example, filtration, extraction, crystallization, different forms of chromatography, including column, thin layer, preparative low or high pressure liquid chromatography, or a combination of these procedures, in addition to other equivalent separation or isolation procedures.

Alternatively, the antisense oligonucleotides of the present invention can be prepared by enzymatic digestion of the naturally occurring target gene by methods known in the art.

Antisense oligonucleotides can also be prepared through the use of recombinant methods. The present invention, therefore encompasses expression vectors comprising nucleic acid sequences that encode the antisense oligonucleotides and expression of the encoded antisense oligonucleotides in a suitable host cell. Such expression vectors can be readily constructed using procedures known in the art. Examples of suitable vectors include, but are not limited to, plasmids, phagemids, cosmids, bacteriophages, baculoviruses and retroviruses, and DNA viruses. One skilled in the art will understand that selection of the appropriate host cell for expression of the antisense oligonucleotide will be dependent upon the vector chosen. Examples of host cells include, but are not limited to, bacterial, yeast, insect, plant and mammalian cells.

One skilled in the art will also understand that the expression vector may further include regulatory elements, such as transcriptional elements, required for efficient transcription of the antisense oligonucleotide sequences. Examples of regulatory elements that can be incorporated into the vector include, but are not limited to, promoters, enhancers, terminators, and polyadenylation signals. The present invention, therefore, provides vectors comprising a regulatory element operatively linked to a nucleic acid sequence encoding an antisense oligonucleotide. One skilled in the art will appreciate that selection of suitable regulatory elements is dependent on the host cell chosen for expression of the antisense oligonucleotide and that such regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian or insect genes.

In the context of the present invention, the expression vector may additionally contain a reporter gene. Suitable reporter genes include, but are not limited to, β-galactosidase, green fluorescent protein, red fluorescent protein, luciferase, and β-glucuronidase. Incorporation of a reporter gene into the expression vector allows transcription of the antisense oligonucleotide to be monitored by detection of a signal generated by expression of the reporter gene.

In accordance with the present invention, the expression vectors can be introduced into a suitable host cell or tissue by one of a variety of methods known in the art. Such methods can be found generally described in Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1992); Ausubel *et al., Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore Maryland (1989); Chang *et al., Somatic Gene Therapy*, CRC Press, Ann Arbor MI (1995); Vega *et al., Gene Targeting*, CRC Press, Ann Arbor, MI (1995); and Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworth's, Boston MA (1988) and include, for example, stable or transient transfection, lipofection, electroporation, and infection with recombinant viral vectors.

In vitro *Testing of the Antisense Oligonucleotides*

The effectiveness of the antisense oligonucleotides of the present invention in inhibiting target gene expression can be demonstrated initially *in vitro*, using for example the method previously described by Choy *et al.* (1998) (*Cancer Res.* 48: 6949-6952)or the methods described in the Examples. The colony forming ability of neoplastic cells treated with different antisense ODNs or ODN combinations can be tested by adding to neoplastic cells

grown to an appropriate density (e.g. approximately 1x 10^4), an appropriate concentration of antisense oligonucleotide in the presence of cationic lipid (e.g. lipofectin to a final concentration of 5 μg/mL), washing away excess antisense ODN after a suitable incubation period and then culturing the cells. Percent inhibition is calculated by comparison of the number of colonies in said culture to the number of colonies in cultures grown without pretreatment with antisense ODNs (as visualized, for example, by methylene blue staining)

In the alternative, the antisense oligonucleotide can be introduced into a cell line that normally expresses the protein encoded by the target gene and the amount of mRNA transcribed from the gene can be measured, for example, by Northern blot analysis. Alternatively, the amount of protein encoded by the target gene, and produced by the cell can be measured, for example, by Western blot analysis. The amount of mRNA or peptide produced in a cell treated with the antisense oligonucleotide can then be compared against the amount produced in a control, untreated cell and will provide an indication of how successfully the antisense oligonucleotide has inhibited target gene expression. Such methods are well known in the art and can be used to select antisense ODN combinations for use in neoplastic cells that overexpress the target mRNA, particularly where such overexpression can be correlated to tumor drug resistance.

***In vivo* and *Ex vivo* Testing of the Antisense Oligonucleotides**

The efficacy of the antisense oligonucleotide combinations of the present invention can be tested *in vivo* in an appropriate animal model. Nude mice models (e.g. CD-1 athymic or N:NIH-*bg-nu-xid* from Charles River Laboratories (QC, Canada) are an examples of a suitable animal model in order to examine the effects of antisense ODNs on the growth of neoplastic cells (e.g. by the measurement of tumor volume/weight) or to measure the metastasis of neoplastic cells as measured by the number of new cancerous lesions that appear in mice after being injected with neoplastic cells. Antisense oligonucleotides may be administered to neoplastic cells *ex vivo* prior to injection of said cells into nude mice or otherwise administered to the mice *in vivo* after the injection of said cells into nude mice.

The antisense oligonucleotides of the present invention can be administered to the animal by, for example, systemic administration (e.g. tail vein injection) or local administration, e.g. into

a tumor. Alternatively, the antisense ODNs can be administered by continuous spinal delivery, for example, via an intrathecal catheter attached to a mini-osmotic pump.

Applications for the Antisense Oligonucleotides

The antisense ODN combinations of the present invention are useful as drugs for the treatment of cancer or proliferative disorders irrespective of their origin. In an embodiment of the invention, the antisense ODN combinations are used to improve cancer therapies in drug resistant cancers which can arise, for example, from heterogeneity of tumor cell populations, alterations in response to chemotherapy and increased malignant potential. Such changes are often more pronounced at advanced stages of disease and have, in part, as an underlying cause changes in genome/message stability.

In another embodiment of the invention, the antisense ODN combinations are used to improve cancer therapies in drug sensitive cancers.

Cancers which may be treated using the methods of the invention, include, but are not limited to carcinomas, leukemias (e.g. of the central-nervous system and blood), lymphomas (Hodgkins and non-Hodgkins), sarcomas, melanomas, adenomas, neuroblastomas, nephroblastomas (e.g. Wilm's tumor) and retinoblastomas.

Examples of carcinomas (i.e. originating in epithelial tissues such as the skin and inner membrane surfaces of the body), include, but are not limited to cancers such as breast cancer, colon cancer, rectal cancer, esophageal cancer, prostate cancer, lung cancer, stomach cancer, bladder cancer, skin cancer, kidney cancer, pancreatic cancer, ovarian cancer, uterine cancer, cervical cancer, cancer of the vulva, liver cancer, thyroid cancer, aveolar cell carcinoma, basal cell carcinoma, bronchogenic carcinoma, chorionic carcinoma, embyronal carcinoma, giant cell carcinoma, glandular carcinoma, medullary carcinoma, melatonic carcinoma, mucinous carcinoma, oat cell carcinoma, scirrhous carcinoma and squamous cell carcinoma.

Examples of sarcomas (i.e. originating in soft tissues of messenchymal origin such as the connective and supportive tissues of muscle, bone, cartilage and fat), include, but are not limited to cancers such as Kaposi's sarcoma, alveolar soft part sarcoma, bone cancer, botryoid

sarcoma, endometrial sarcoma, giant cell sarcoma, osteogenic sarcoma, reticulum cell sarcoma and spindle cell sarcoma, rhabdomyosarcoma and lymphosarcoma.

In the treatment of cancer, the antisense ODN combinations may be used in conjunction with a variety of chemotherapeutic agents, particularly for the treatment of cancers suspected of being drug resistant. Chemotherapeutic agents (e.g. synthetic chemical medications) have toxic effects that selectively or non-selectively destroy cancerous tissue. Examples of TS inhibiting chemotherapeutics include, but are not limited to 5-FU, 5-FRdR, raltitrexed, methotrexate, capecitabine (an oral form of 5-FU), Alimta® and a topical 5-FU cream (Effudex®). Antisense ODN combinations can also have an at least additive effect with other chemotherapeutics, including, but not limited to hydroxyurea, Tesmilifene®, busulphan, cisplatin, cyclophosphamide, daunorubicin, doxorubicin, melphalan, vincristine, vinblastine, Navelbine® and chlorambucil. Antisense ODN combinations can also have an at least additive effect when used in conjunction with radiotherapy.

These medications are generally administered using a particular therapeutic egimen over a period of weeks or months, but can also have deleterious effects on healthy tissues, e.g. by suppressing the bone marrow to some degree or lowering white blood cell counts, resulting in increased risk of infection for patients due to immunosuppression. Other chemotherapeutic agents such as vincristine, which has proved particularly useful as an intravenously administered oncolytic agent in combination with other oncolytic agents for the treatment of various cancers can bring about, following single weekly doses, a wide range of adverse reactions including hair loss, leukopenia, neuritic pain, constipation, and difficulty in walking, abdominal cramps, ataxia, foot drop, weight loss, optic atrophy with blindness, transient cortical blindness, fever, cranial nerve manifestations, parehesia and numbness of the digits, polyuria, dysuria, oral ulceration, headache, vomiting, diarrhea, and intestinal necrosis and/or perforation. Studies on adverse reactions based on use of Navelbine™ as a single agent indicate Granculocytopenia as the major dose-limiting toxicity, although it was generally reversible and not cumulative over time. Mild to moderate peripheral neutopathy manifested by pareathesia and hypesthesia are the most frequently reported neurologic toxicities, occurring in 10% of patients. Mild to moderate nausea occurs in roughly one-third of patients treated with Navelbine™ with a slightly lesser fraction experiencing constipation, vomiting, diarrhea, anorexia, and stomatitis.

While a few compounds exhibiting lessened toxic effects with equal or greater chemotherapeutic activity have been achieved, e.g. 3',4'-anhydrovinblastine as compared to other vinca alkaloids, the process of synthesizing such chemical medicines and screening them for activity can be labour and time intensive. Thus a need remains for relatively non-toxic drugs that can be produced efficiently and provide improved anti-tumour efficacy for the treatment of cancer, or otherwise potentiate the effect of known chemotherapeutics. Consequently the present invention provides for a method of reducing the deleterious effects of chemotherapeutic agents using antisense ODN combinations directed against the same TS mRNA. Such antisense ODNs are less toxic and reduce the amount of chemotherapeutic agent required to elicit an effective anti-cancer response, thereby reducing the incidence of undesirable side effects in patients. For example, in a specific embodiment of the invention TS antisense ODN SEQ ID NO: 1 is known not to exhibit overt toxicity in animals (Berg *et al.*, 2001).

Standard dosage and administration regimens for chemotherapeutic agents are well known in the art and can be found, for example, in the product monographs published in the Compendium of Pharmaceuticals and Specialties, 31st Edition, 1996 (CPS), or the latest edition thereof.

For example, Navelbine® is indicated for the treatment of cancer or tumor such as breast cancer and non-small cell lung cancer and the dosage and administration protocols suggested in the product monograph in the CPS is 30mg/m^2, administered weekly *via* intravenous injection over 6 to 10 minutes. No dose adjustment is required for patients with renal insufficienty. Adjustment to dosage is suggested in accordance with hematologic toxicity or hepatic insufficiency. As another example, the use of Tesmilifene® in the treatment of prostate cancer, particularly hormone-unresponsive metastatic prostate cancer, is administered as an initial intravenous infusion of Tesmilifene® over an approximately one hour period prior to cyclophosphamide treatment, has been shown to potentiate the anti-cancer activity and ameliorate the toxicity associated with using the chemotherapeutic cyclophosphamide or another normally substantially inactive agent. A daily dose of about 240 to about 1200 mg/M^2 of Tesmilifene® has been shown to afford maximum bone marrow protection and synergy with chemotherapy to kill cancer cells. (US5863912)

5-FU has been used as chemotherapeutic for many years alone and in conjunction with other chemotherapeutics. The following exemplary therapeutic regimens are provide with the understanding that one skilled in the art would appreciate that they may be applied to the situations where 5-FU is used alone or conjunction with another chemotherapeutic. A first exemplary regimen is the Mayo regimen, wherein 5-FU is administered at 425 mg/m^2 by intravenous bolus injection daily together with 20 mg/m^2 leucovorin for 5 days , followed by 3 weeks off. A second therapeutic regimen may consist of administering 200 to 220 mg/m^2 5-FU by continuous infusion over 24 hours once a week. A third means of administering 5-FU consists of using an oral version of the drug, capecitabine, i.e. Xeloda® in two divided doses for a total of 2000-2500 mg/m^2 daily. A fourth therapeutic regimen consists of shorter, intermittant infusions of 5-FU from between 24 to 120 hours, every weekly, two weeks, three weeks or every four every weeks at dosages of 600 mg/m^2 to 2500 mg/m^2 per 24 hours. It is contemplated that the antisense ODN combinations directed against TS mRNA allow for the amount of such chemotherapeutic as described in the above exemplary regimens to be reduced while still preserving a substantially equivalent anti-tumor effect. It is also contemplated that the antisense ODN combinations directed against TS mRNA allow for the enhancement of the efficacy of the above standard protocols. On skilled in the art also appreciates that 5'FU and its variants can be used in conjunction with a variety of other traditional chemotherapeutic drugs.

An exemplary therapeutic regimen for raltitrexed (Tomudex®) is administration at 3 mg/m^2 once every 3 weeks by bolus injection.

It is contemplated that antisense ODN combinations directed against TS mRNA have application against advanced neoplastic (i.e. overt disease in a patient, wherein such overt disease is not amenable to cure by local modalities of treatment, such as surgery or radiotherapy). It is therefore further contemplated that use of antisense oligonucleotide combinations directed against TS mRNA will allow for more prolonged treatments as may be necessary to treat advanced disease. It is also contemplated that antisense ODN combinations directed against TS mRNA have application as part of adjuvant therapies where the intention is to cure the cancer in a patient.

Pharmaceutical Preparations Antisense Oligonucleotide Combinations

When employed as pharmaceuticals, the antisense oligonucleotides are usually administered in the form of pharmaceutical compositions or formulations. The antisense ODN combinations are generally mixed together in the same composition but may also be administered simultaneously as separate compositions. Such compositions are prepared in a manner well known in the pharmaceutical art and comprise at least one active compound. In a related embodiment, the pharmaceutical composition or formulation comprises a vector encoding the antisense oligonucleotide of the present invention.

In accordance with the present invention, the antisense oligonucleotides may be incorporated into pharmaceutical compositions in the form of pharmaceutically acceptable salts. The term "pharmaceutically acceptable salt" as used herein refers to salts which retain the biological effectiveness and properties of the antisense oligonucleotides of the present invention, and which are not biologically or otherwise undesirable. In many cases, the antisense oligonucleotides of the present invention are capable of forming acid and/or base addition salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

Pharmaceutically acceptable base addition salts can be prepared from inorganic and organic bases. Salts derived from inorganic bases include, but are not limited to, sodium, potassium, lithium, ammonium, calcium and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary and tertiary amines, such as alkyl amines, dialkyl amines, trialkyl amines, substituted alkyl amines, di(substituted alkyl) amines, tri(substituted alkyl) amines, alkenyl amines, dialkenyl amines, trialkenyl amines, substituted alkenyl amines, di(substituted alkenyl) amines, tri(substituted alkenyl) amines, cycloalkyl amines, di(cycloalkyl) amines, tri(cycloalkyl) amines, substituted cycloalkyl amines, disubstituted cycloalkyl amine, trisubstituted cycloalkyl amines, cycloalkenyl amines, di(cycloalkenyl) amines, tri(cycloalkenyl) amines, substituted cycloalkenyl amines, disubstituted cycloalkenyl amine, trisubstituted cycloalkenyl amines, aryl amines, diaryl amines, triaryl amines, heteroaryl amines, diheteroaryl amines, triheteroaryl amines, heterocyclic amines, diheterocyclic amines, triheterocyclic amines, mixed di- and tri-amines where at least two of the substituents on the amine are different and are selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, heteroaryl, heterocyclic,

and the like. Amines in which two or three substituents, together with the amino nitrogen, form a heterocyclic or heteroaryl group are also suitable.

Examples of suitable amines include, but are not limited to, isopropylamine, trimethyl amine, diethyl amine, tri(*iso*-propyl) amine, tri(*n*-propyl) amine, ethanolamine, 2-dimethylaminoethanol, tromethamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, N-alkylglucamines, theobromine, purines, piperazine, piperidine, morpholine, N-ethylpiperidine, and the like. It should also be understood that carboxylic acid derivatives would be useful in the practice of this invention, for example, carboxylic acid amides, including carboxamides, lower alkyl carboxamides, dialkyl carboxamides, and the like.

Pharmaceutically acceptable acid addition salts can be prepared from inorganic and organic acids. Salts derived from inorganic acids include hydrochloric acid, hydrobromic acid, sulphuric acid, nitric acid, phosphoric acid, and the like. Salts derived from organic acids include acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulphonic acid, ethanesulphonic acid, *p*-toluene-sulphonic acid, salicylic acid, and the like.

Administration of the Antisense Oligonucleotides

The antisense oligonucleotide combinations of the present invention and pharmaceutical compositions comprising same may be administered in a number of ways depending upon whether local or systemic treatment of the organism is desired. Administration may be pulmonary, *e.g.* by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, *e.g.* intrathecal or intraventricular, administration. The antisense oligonucleotide combinations of the present invention and pharmaceutical compositions comprising same may be administered topically in a lotion or cream, for application to the skin in order to treat for example a melanoma.

The antisense oligonucleotide combinations of the present invention may be delivered alone one after the other or in combination simultaneously, and may be delivered along with a pharmaceutically acceptable vehicle. Ideally, such a vehicle would enhance the stability and/or delivery properties. The present invention also provides for administration of the antisense oligonucleotides or pharmaceutical compositions comprising the antisense oligonucleotides using a suitable vehicle, such as a liposome, microparticle or microcapsule. In various embodiments of the invention, the use of such vehicles may be beneficial in achieving sustained release of the active component, or otherwise protecting the antisense ODNs from nuclease degradation.

For administration to an individual for the treatment cancer, the present invention also contemplates the formulation of the antisense oligonucleotides or pharmaceutical compositions comprising the antisense oligonucleotides into oral dosage forms such as tablets, capsules and the like. For this purpose, the antisense oligonucleotides or pharmaceutical compositions comprising the antisense oligonucleotides can be combined with conventional carriers, such as magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethyl-cellulose, low melting wax, cocoa butter and the like. Diluents, flavoring agents, solubilizers, lubricants, suspending agents, binders, tablet-disintegrating agents and the like can also be employed, if required. The antisense oligonucleotides or pharmaceutical compositions comprising the antisense oligonucleotides can be encapsulated with or without other carriers. In all cases, the proportion of active ingredients in any solid and liquid composition will be at least sufficient to impart the desired activity to the individual being treated upon oral administration. The present invention further contemplates parenteral injection of the antisense oligonucleotides or pharmaceutical compositions comprising the antisense oligonucleotides, in which case they are used in the form of a sterile solution containing other solutes, for example, enough saline or glucose to make the solution isotonic.

For administration by inhalation or insufflation, the antisense oligonucleotides or pharmaceutical compositions comprising the antisense oligonucleotides can be formulated into an aqueous or partially aqueous solution, which can then be utilized in the form of an aerosol.

The present invention also provides for administration of the antisense oligonucleotides in the form of genetic vector constructs that are designed to direct the *in vivo* synthesis of the antisense oligonucleotides. Within the vector construct, the nucleic acid sequence encoding the antisense oligonucleotide is under the control of a suitable promoter. The vector construct may additionally contain other regulatory control elements. Methods of constructing and administering such genetic vector constructs for *in vivo* synthesis of antisense oligonucleotides are well-known in the art. U.S. Patent No. 6,265,167 teaches an efficient method for the introduction, expression and accumulation of antisense oligonucleotides in the cell nucleus. This method allows the antisense oligonucleotide to hybridize to the sense mRNA in the nucleus, and thereby prevents the antisense oligonucleotide being either processed or transported into the cytoplasm.

The dosage requirements for the antisense oligonucleotides of the present invention or pharmaceutical compositions comprising the antisense oligonucleotides vary with the particular compositions employed, the route of administration, the severity of the symptoms presented and the particular subject being treated. Dosage requirements can be determined by standard clinical techniques, known to a worker skilled in the art. Treatment will generally be initiated with small dosages less than the optimum dose of the compound. Thereafter the dosage is increased until the optimum effect under the circumstances is reached. In general, the antisense oligonucleotides or pharmaceutical compositions comprising the antisense oligonucleotides are administered at a concentration that will generally afford effective results without causing any harmful or deleterious side effects. Administration can be either as a single unit dose or, if desired, the dosage can be divided into convenient subunits that are administered at suitable times throughout the day.

In an embodiment of the invention it is contemplated that antisense ODN combination directed against the same target mRNA is administered at a total concentration equal to or less than an equivalent effective amount of any single ODN used alone. In another embodiment the invention, the amount of antisense ODN combination is administered at a total concentration sufficient to chemosensitize cancer cells to a chemotherapeutic agent to an extent at least as much as an equivalent effective amount of the more potent/active ODN of the combination used alone. In a specific embodiment the chemotherapeutic agent is

administered within 4 to 24 hours of treatment with an antisense ODN combination of the invention. In another specific embodiment antisense ODN combination are systemically administered to patients, for example, by bolus injection or contiuous infusion into a patient's bloodstream.

Therapeutic Uses and Strategies

The antisense oligonucleotide combinations of the invention may be used as part of a neo-adjuvant therapy (to primary therapy), as part of an adjuvant therapy regimen, or also for the treatment of locally advanced and metastatic disease.

Primary therapy is understood to encompass a first line of treatment upon the initial diagnosis of cancer in a patient. Exemplary primary therapies may involve surgery, a wide range of chemotherapies and radiotherapy.

Adjuvant therapy is understood to encompass any therapy, following a primary therapy such as surgery, that is administered to patients at risk of relapsing. Adjuvant systemic therapy is begun soon after primary therapy to delay recurrence, prolong survival or cure a patient. One kind of adjuvant systemic therapy is adjuvant chemotherapy, e.g., using 5-fluorouracil alone or in combination with methotrexate for breast and colorectal cancers, over the course of e.g., four to 24 months. It is contemplated that the antisense ODN combinations can be used in further combination with other chemotherapeutic agents as part of an adjuvant therapy.

In the application of cancer therapies a patient's response status is monitored and refers to measuring what happens to the tumour(s) or lesion(s) under chemotherapy, namely any observed growth (progression of disease), stability, or shrinkage (complete or partial response). Arising out such monitoring may be the observation of relapse in a patient which may refer to the relapse of a patient with advanced disease. Relapse time is the time from the initial appearance of a primary cancer to the appearance of advanced disease requiring chemotherapy.

The progression of advanced disease is monitored to help evaluate when chemotherapy, may be appropriate and may be marked by an increase of at least 25% in the overall sum of measurable lesions as compared to nadir (*i.e.* best response) and/or the appearance of new lesions following primary therapy. Alternatively, lesions may be found to shrink in size.

Kits

The present invention additionally provides for therapeutic kits containing one or more antisense ODNs that may used in combination, or one or more expression vectors encoding the antisense ODNs, in pharmaceutical compositions for use in the treatment of cancer. The contents of the kit can be lyophilized and the kit can additionally contain a suitable solvent for reconstitution of the lyophilized components. Individual components of the kit would be packaged in separate containers and, associated with such containers, can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

When the components of the kit are provided in one or more liquid solutions, the liquid solution can be an aqueous solution, for example a sterile aqueous solution. For *in vivo* use, the expression construct may be formulated into a pharmaceutically acceptable syringeable composition. In this case the container means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the animal, such as the lungs, injected into an animal, or even applied to and mixed with the other components of the kit.

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

Cell Culture and Chemicals

HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. All tissue culture reagents, including LipofectAmine® 2000 (LFA 2000), were from Invitrogen Canada (Burlington, ON, Canada). Raltitrexed (AstraZeneca, Macclesfield, UK) was dissolved in 0.1 M sodium bicarbonate, and 5-FUdR (Sigma, St. Louis, MO) was dissolved in water, and diluted in serum-free medium prior to use. The use of LFA+ as opposed to LFA doubled the activity of ODN combinations SEQ ID NO: 1 + 2 and 1 + 3 observed *in vitro*.

Oligodeoxynucleotides

Fully phosphorothioated ODNs with 2'-methoxy-ethoxy modification on the 6 nucleotides at both the 5'- and 3'-ends were generously provided by ISIS Pharmaceuticals, Carlsbad, CA. ODN SEQ ID NO: 1 (5'-GCCAGTGGCAACATCCTTAA-3'), ODN SEQ ID NO: 5 (5'-CCAGCCCAACCCCTAAAGAC-3'), and ODN SEQ ID NO: 3 (5'-ACTCAGCTCCCTCAGATTTG-3') are complementary to nucleotides 1184-1203, 1081-1100, and 1436-1455, respectively, in the 3' untranslated region of human TS, while ODN SEQ ID NO: 6 (5'-GGCATCCCAGATTTTCACTC-3') and ODN SEQ ID NO: 2 (5'-TTGGATGCGGATTGTACCCT-3') are complementary to nucleotides 380-399, 419-438, and 1002-1021, respectively, within the protein coding region. The scrambled control ODN SEQ ID NO: 4 (5'-ATGCGCCAACGGTTCCTAAA-3') has the same base composition as ODN SEQ ID NO: 1, in random order, and is not complementary to any region of human TS. There are no other known human mRNAs with more than 15 bases of complementarity to any of the ODNs used. The ODNs were diluted in Milli-Q purified water, and concentrations calculated based on spectrophotometric absorbance readings.

Antisense ODN and Chemotherapeutic Drug Treatments

HeLa cells were plated at 7.5×10^4 cells per 25-cm^2 flask in 2 ml of medium. On the following day, the required amounts of ODN and LipofectAmine 2000 were pre-mixed for 15 minutes in 5 to 10 ml of serum-free medium. Complete medium was added to yield a 2× transfection mix, and 2 ml of this mixture was added to each flask. Cytotoxic drugs were added to the flasks 4 hours after ODN treatment, in 100 µl volumes. In some experiments, a 1× transfection mix was prepared, and the culture medium on the cells was replaced with 2 ml of the 1× mixture. In this case, cytotoxic drugs were diluted in growth medium to 2× final concentration and added in 2 ml aliquots after 4 hours, and therefore the concentration of ODN was reduced at that time. The ODN concentrations reported indicate the initial ODN concentration, representing the 4 hour pre-treatment values. Similar results were obtained with each method.

Cells were counted using a Coulter counter (Beckman Coulter, Hialeah, FL) on the day of treatment, and 4 days later. Proliferation is expressed as a percentage of that in control flasks using the formula: 100 × (experimental final cell number – initial cell number) ÷ (control final cell number – initial cell number).

RNA Preparation and Analysis

For isolation of RNA, cells were plated at 1×10^6 cells per 75-cm^2 flask in 5 ml of medium. On the following day, ODNs (600 nM) were mixed with 6 µg/ml lipid in serum-free medium for 15 minutes at room temperature to yield a 6× transfection mix. One mL of the ODN:lipid mixture was added to each 75-cm^2 flask to yield final concentrations of 100 nM ODN and 1 µg/ml lipid. RNA was prepared from HeLa cells using the TRIzol reagent (Invitrogen Canada), and quantified using a spectrophometer. For reverse-transcription polymerase chain reaction (RT-PCR), cDNA was prepared from 1 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen Canada) and random hexamer primers. Two percent of the cDNA produced was used as template for PCR with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers GAP-for (5'-TATTGGGCGCCTGGTCACCA-3'; SEQ ID NO: 7) and GAP-rev (5'-CCACCTTCTTGATGTCATCA-3'; SEQ ID NO: 8), or the TS primers TS-for (5'-TTTTGGAGGAGTTGCTGTGG-3'; SEQ ID NO: 9) and TS-rev (5'-TGTGCATCTCCCAAAGTGTG-3'; SEQ ID NO: 10). PCR cycling parameters were: 3 minutes at 94°C; followed by 24 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 45 seconds at 72°C; and a 7 minute 72°C extension. Products were resolved on 1.5% agarose

gels and stained with ethidium bromide. Quantitation of images captured using the ImageMASTER VDS gel documentation system (Amersham Pharmacia Biotech) was done with ImageQuant version 5.1 (Molecular Dymamics).

TS Protein Quantitation

The assay was adapted (Ferguson *et al.*, 1999) from a published 5-fluorodeoxyuridine monophosphate (5-FdUMP) binding assay (Spears and Gustavsson, 1988). Cell lysates were prepared by freezing, thawing, and sonication in 100 mM potassium phosphate, pH 7.4. Following centrifugation at 6500*g* for 30 minutes at 4°C, protein concentrations in the supernatants were determined using a protein assay kit (Bio-Rad, Hercules, CA). Aliquots containing 100 μg of total protein were incubated with 75 μM methylene tetrahydrofolate, 100 mM 2-mercaptoethanol, and 15 nM [6-^{3}H]5-FdUMP (specific activity 18.6 Ci/mmol, Moravek Biochemicals, Brea, CA), in 50 mM potassium phosphate (pH 7.4) for 30 minutes at 37°C. Reactions were stopped by addition of 1 ml of albumin-coated, acidified charcoal and, after 10 minutes at room temperature, centrifuged two times at 5000*g* for 30 min at 22°C to remove particulate matter. [6^{3}H]-5-FdUMP bound to TS, and therefore unavailable for precipitation with charcoal, was quantitated by scintillation counting.

Flow Cytometry

Cells were treated with 200 nM ODNs and 2 μg/ml lipid, in the same manner as described for RNA preparation, and collected by trypsinization at 48 hours after ODN treatment. Cells were washed with phosphate-buffered saline, fixed in 75% ethanol for 15 minutes at room temperature, and washed again. DNA was stained with propidium iodide (0.02 mg/ml in phosphate-buffered saline with 0.1% (vol/vol) Triton X-100 and 0.2 mg/ml deoxyribonuclease-free ribonuclease A) and analysed on a Beckman Coulter XL-MCL flow cytometer. At least 50,000 single cells were analysed for each condition, and the distribution of cells in G0/G1, S, and G2/M cell cycle phases was calculated using MultiCycle (Version 3.0) software (Phoenix Flow Systems, San Diego, CA).

Statistical Analysis

Statistical significance within experiments was determined using Student's *t* test ($p<0.05$). All experiments were repeated at least twice.

EXAMPLE 1:

ODN Combinations Enhance Antisense Effects on TS mRNA and Protein Levels and Proliferation of HeLa Cells

To test whether the combinations of antisense ODNs targeting the same mRNA might be more effective than single ODNs, several pairs of antisense ODNs targeting human TS mRNA were analyzed for their antiproliferative effects on HeLa cells *in vitro*. Figure 1A shows one TS antisense ODN that did not augment the ability of ODN SEQ ID NO: 1 to inhibit proliferation, while several combinations of antisense ODNs significantly increased the antiproliferative activity compared with an equimolar mixture of ODN SEQ ID NO: 1 plus the scrambled control ODN SEQ ID NO: 4. Activity of several equimolar (50 nM plus 50 nM) mixtures was also significantly greater than 100 nM of ODN SEQ ID NO: 1 alone, whereas varying the ratios from 1:7 to 7:1 did not further augment the enhanced activity (data not shown). Two combinations, namely ODNs SEQ ID NOs: 1 + 2 and ODNs SEQ ID NOs: 1 + 3, demonstrated enhanced antiproliferative activity when compared to either ODN alone, over a range of concentrations (Figure 1B and 1C). The combination of ODNs SEQ ID NOs: 2 + 3 was equivalent to ODN SEQ ID NO: 3 alone, and the triple mixture was no more effective than either double mixture (data not shown).

RT-PCR analysis indicated that these antisense ODN combination treatments resulted in enhanced reductions of TS mRNA levels, compared to treatment with each ODN used as a single agent (Figure 2A and 2B). Decreased TS mRNA levels were reflected by reductions in TS protein levels, as measured by [6^3H]5-FdUMP binding (Figure 2C). Flow cytometry analysis indicated that treatment with ODN SEQ ID NO: 1 alone or with the combination of ODNs SEQ ID NOs: 1 + 2 induced G2/M arrest to a similar extent, but that ODN SEQ ID NO: 2 alone had no significant effect on the cell cycle profile (Figure 3).

EXAMPLE 2:

Antisense ODN Combinations Chemosensitize HeLa Cells to Anti-TS Drugs

The ability of these combinations of ODNs to enhance the sensitivity of HeLa cells to the TS inhibitors raltitrexed and 5-FUdR was examined. Treatment with the combinations of ODNs SEQ ID NOs: 1 + 2 or ODNs SEQ ID NOs: 1 + 3 increased the cytotoxicity of raltitrexed and 5-FUdR, but only to the same extent as treatment with ODN SEQ ID NO: 1 alone (Figure 4).

The slightly increased cytotoxicity of 0.33 and 0.66 nM raltitrexed following treatment with ODNs SEQ ID NOs: 1 + 2 trended to effect ($p = 0.0615$ and 0.0739, respectively). ODN SEQ ID NO: 2 is representative of several other TS antisense ODNs that had little or no dose-dependent effects on proliferation when used as single agent treatments, but effectively enhanced the cytotoxicity of raltitrexed and 5-FUdR (data not shown). ODN SEQ ID NO: 2 is representative of TS antisense ODNs that enhance proliferation when used on its own, but which also effectively enhances the cytotoxicity of 5-FudR.

HeLa cells were also treated with varying concentrations of ODN SEQ ID NO. 1, followed by various doses of raltitrexed or 5-FUdR. The cytotoxicities of raltitrexed and 5-FUdR were significantly increased at all of the tested doses of ODN SEQ ID NO. 1. Treatment with ODN SEQ ID NO. 1 at the lowest dose tested (12.5 nM) effectively sensitized HeLa cells to the cytotoxicity of raltitrexed, and while the cytotoxicity of 5-FUdR was increased at that concentration, maximal chemosensitivity was achieved at 25 nM ODN SEQ ID NO: 1 (Figure 5). In contrast, a 50 nM dose of ODN SEQ ID NO: 1 is required to significantly inhibit cell proliferation in the absence of chemotherapeutic drug in these experiments, by approximately 20%.

The combination ODN strategy was then applied to sensitize HeLa cells to 5-FUdR cytotoxicity using lower concentrations of ODNs. The combination of ODNs SEQ ID NOs: 1+ 3 at 12.5 nM was equivalent to ODN SEQ ID NO: 1 alone at 25 nM. As shown in Figure 6, equimolar combination of ODNs SEQ ID NOs: 1 + 2 (6.25 nM or 12.5 nM each) significantly augmented chemosensitization to 5-FUdR compared to that achieved with 12.5 or 25 nM of the individual ODNs, respectively.

Summary

Antisense ODN combinations directed against TS mRNA were shown to have a more than additive effect in reducing proliferation compared to the individual ODNs in the absence of drug, but did not increase chemosensitization in a similar manner. It was determined that the synergistic antiproliferative effect of the combinations seems to require a higher concentration of antisense ODN; single ODN treatments at 25, 50, and 100 nM were effective. In contrast, the ODN combinations enhanced chemosensitization relative to single ODN treatments at the 12.5 and 25 nM total doses of ODNs, but not at higher concentrations.

Very low anti- TS ODN combination concentrations sensitize HeLa cells to very low drug concentrations *in vitro*, indicating that appropriate antisense ODN combination design can result in delivery of sufficient levels antisense drug to have a desired chemosensitizing effect even if only sub-optimal penetration of ODNs into a solid tumor is achieved.

In a specific embodiment of the invention antisense ODN combinations increase the effectiveness of reduced doses of cytotoxic drug, and can be used to diminish or eliminate systemic toxicity. The individual antisense ODNs of the combinations (as well as the combinations) have the ability to decrease the amount of TS mRNA and protein in neoplastic cells, as well as chemosensitize neoplastic cells, irrespective of their antiproliferative abilities or effects on cell cycle progression. For example, while ODN SEQ ID NO: 1 treatment induces G2/M cell cycle arrest, inhibits HeLa and HT29 cell proliferation, and enhances the cytotoxicity of TS-targeting chemotherapeutic drugs (Berg *et al.*, 2001; Ferguson *et al.*, 1999), treatment with ODN SEQ ID NO: 2 as a single agent has no apparent effects on the cell cycle profile or on proliferation of HeLa cells at any dose up to 200 nM, yet treatment with only 25 or 50 nM specifically reduces TS mRNA and protein levels and, importantly, sensitizes HeLa cells to TS-inhibitory chemotherapeutic drugs.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A combination of two or more antisense oligonucleotides complementary to a thymidylate synthase mRNA for use in the treatment of cancer together with one or more chemotherapeutic agents, wherein the use of the combination enhances the anti-tumor effect of standard doses of the one or more chemotherapeutic agents.

2. A combination of two or more antisense oligonucleotides complementary to different regions of a thymidylate synthase mRNA for use in the treatment of cancer together with a chemotherapeutic agent, wherein the use of the combination reduces the amount of chemotherapeutic required to effectively treat a mammal with cancer.

3. A combination of two or more antisense oligonucleotides complementary to different regions of a thymidylate synthase mRNA for use together with a chemotherapeutic agent to treat a mammal, wherein the combination and chemotherapeutic agent reduce the number of neoplastic cells in said mammal.

4. A combination of two or more antisense oligonucleotides for use in the treatment of cancer together with one or more chemotherapeutic agents to treat a mammal, wherein the sequences of two oligonucleotides of the combination are selected from the group consisting of SEQ ID NO: 1 and 2, or SEQ ID NO: 1 and 3.

Application number/ Numéro de demande : 2380970

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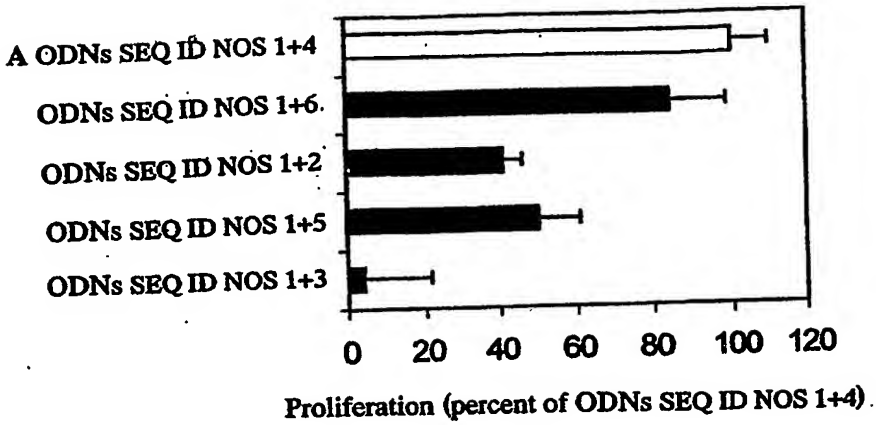
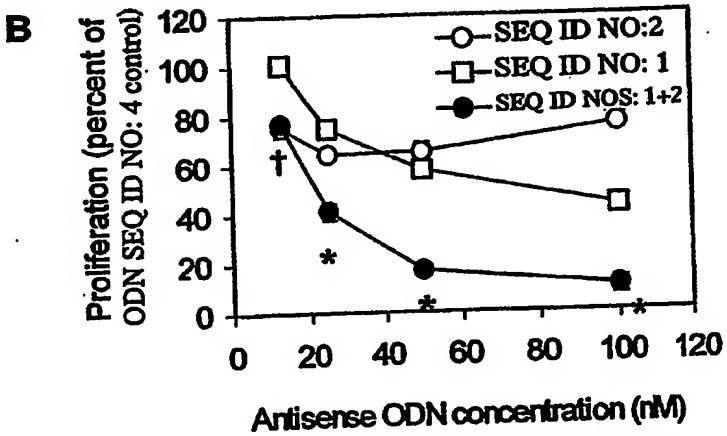
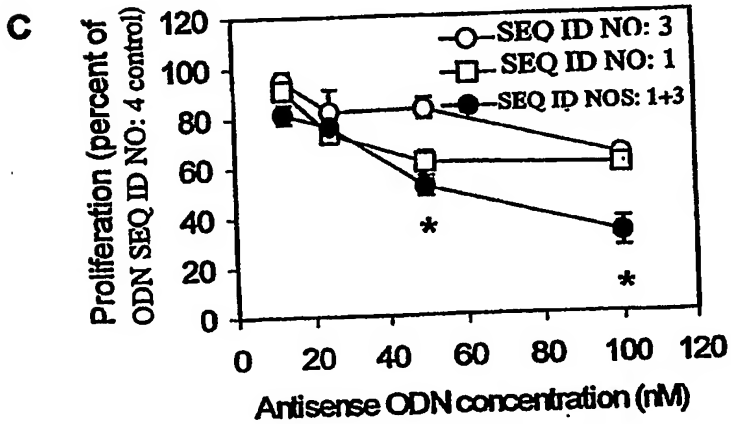
Fig. 1


A ODNs SEQ ID NOS 1+4
ODNs SEQ ID NOS 1+6.
ODNs SEQ ID NOS 1+2
ODNs SEQ ID NOS 1+5
ODNs SEQ ID NOS 1+3
0 20 40 60 80 100 120
Proliferation (percent of ODNs SEQ ID NOS 1+4)


B
Proliferation (percent of ODN SEQ ID NO: 4 control)
120
100
80
60
40
20
0
SEQ ID NO:2
SEQ ID NO: 1
SEQ ID NOS: 1+2
†
*
*
*
0 20 40 60 80 100 120
Antisense ODN concentration (nM)


C
Proliferation (percent of ODN SEQ ID NO: 4 control)
120
100
80
60
40
20
0
SEQ ID NO: 3
SEQ ID NO: 1
SEQ ID NOS: 1+3
*
*
0 20 40 60 80 100 120
Antisense ODN concentration (nM)

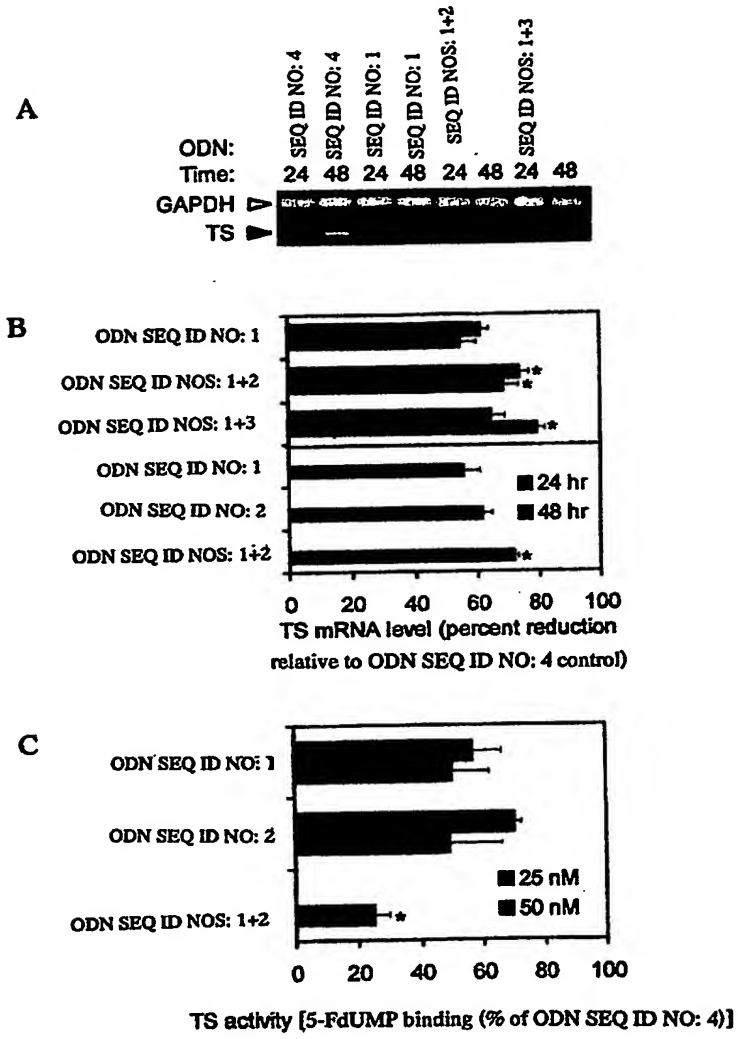
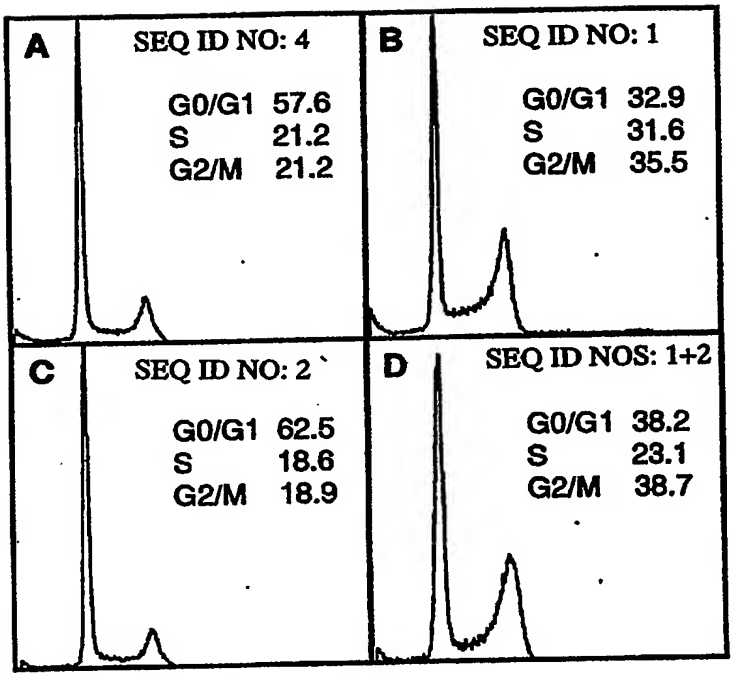

A
SEQ ID NO: 4
SEQ ID NO: 4
SEQ ID NO: 1
SEQ ID NO: 1
SEQ ID NOS: 1+2
SEQ ID NOS: 1+3
ODN:
Time: 24 48 24 48 24 48 24 48
GAPDH
TS
B
ODN SEQ ID NO: 1
ODN SEQ ID NOS: 1+2
ODN SEQ ID NOS: 1+3
ODN SEQ ID NO: 1
ODN SEQ ID NO: 2
ODN SEQ ID NOS: 1+2
24 hr
48 hr
0 20 40 60 80 100
TS mRNA level (percent reduction relative to ODN SEQ ID NO: 4 control)
C
ODN SEQ ID NO: 1
ODN SEQ ID NO: 2
ODN SEQ ID NOS: 1+2
25 nM
50 nM
0 20 40 60 80 100
TS activity [5-FdUMP binding (% of ODN SEQ ID NO: 4)]

Figure 2

Fig. 3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G0/G1 57.6
S 21.2
G2/M 21.2
B
SEQ ID NO: 1
G0/G1 32.9
S 31.6
G2/M 35.5
C
SEQ ID NO: 2
G0/G1 62.5
S 18.6
G2/M 18.9
D
SEQ ID NOS: 1+2
G0/G1 38.2
S 23.1
G2/M 38.7

Fig. 4

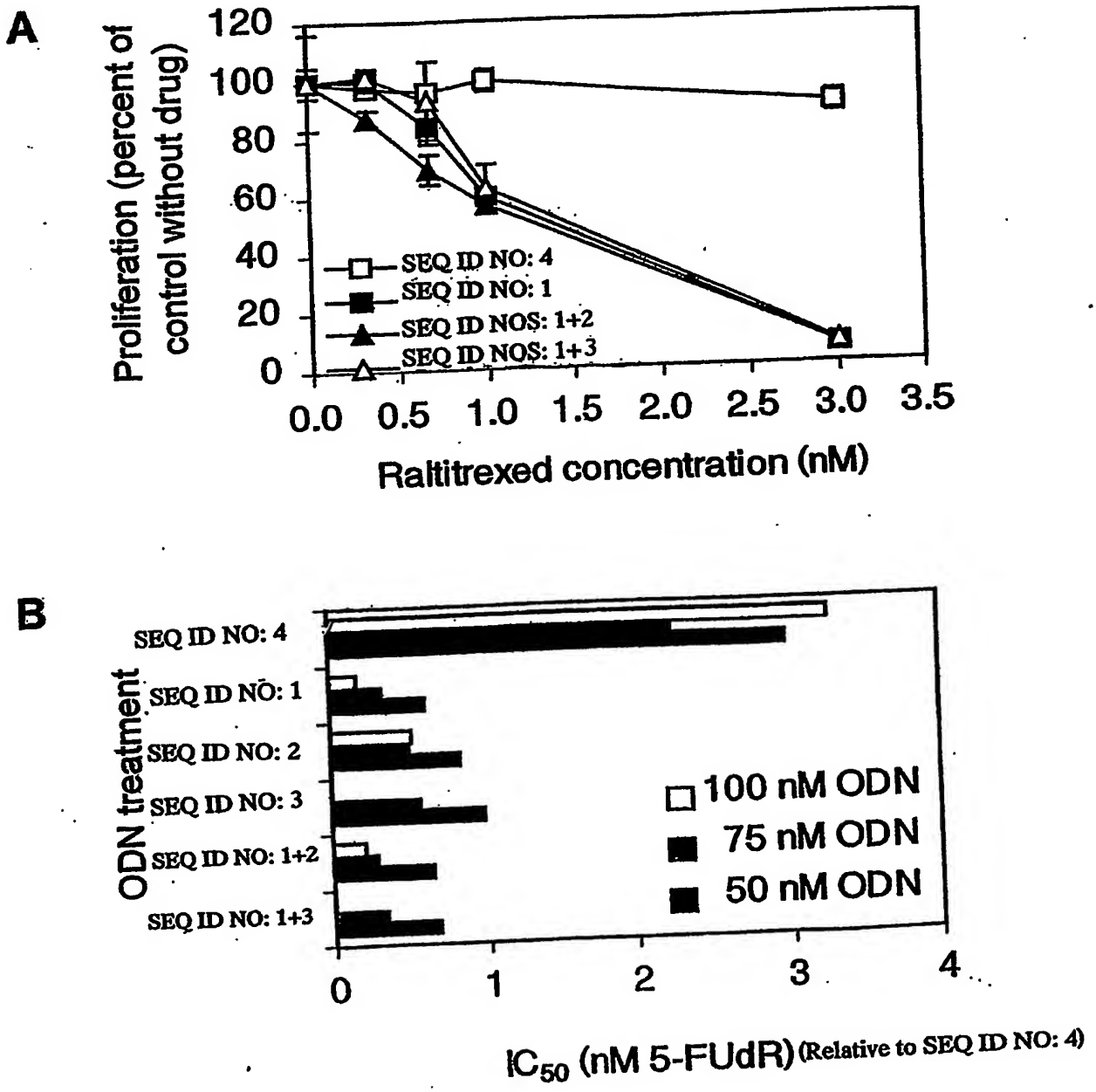
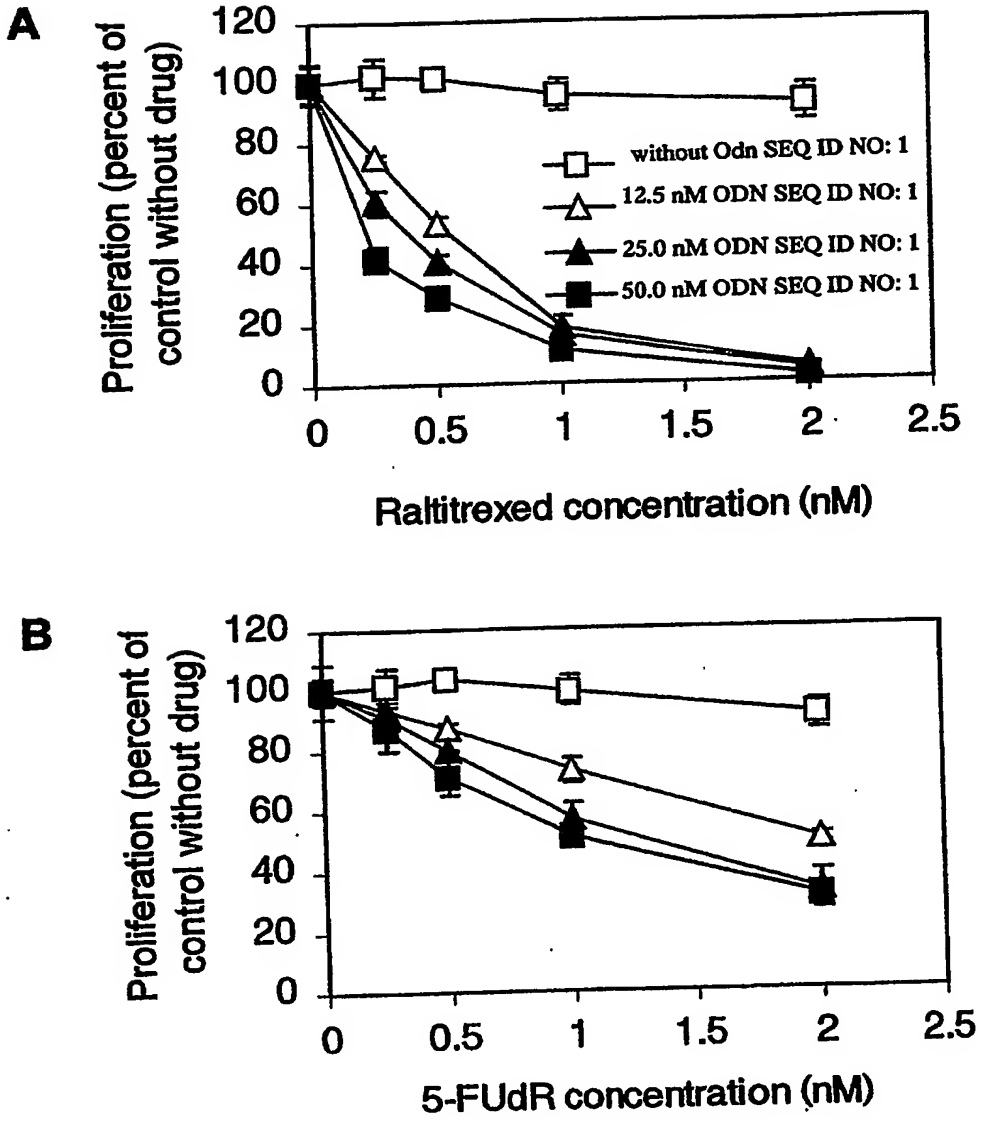

A
Proliferation (percent of control without drug)
120
100
80
60
40
20
0
SEQ ID NO: 4
SEQ ID NO: 1
SEQ ID NOS: 1+2
SEQ ID NOS: 1+3
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
Raltitrexed concentration (nM)
B
ODN treatment
SEQ ID NO: 4
SEQ ID NO: 1
SEQ ID NO: 2
SEQ ID NO: 3
SEQ ID NO: 1+2
SEQ ID NO: 1+3
100 nM ODN
75 nM ODN
50 nM ODN
0 1 2 3 4
IC50 (nM 5-FUdR) (Relative to SEQ ID NO: 4)

Fig. 5


A
Proliferation (percent of control without drug)
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Raltitrexed concentration (nM)
without Odn SEQ ID NO: 1
12.5 nM ODN SEQ ID NO: 1
25.0 nM ODN SEQ ID NO: 1
50.0 nM ODN SEQ ID NO: 1
B
Proliferation (percent of control without drug)
120
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
5-FUdR concentration (nM)

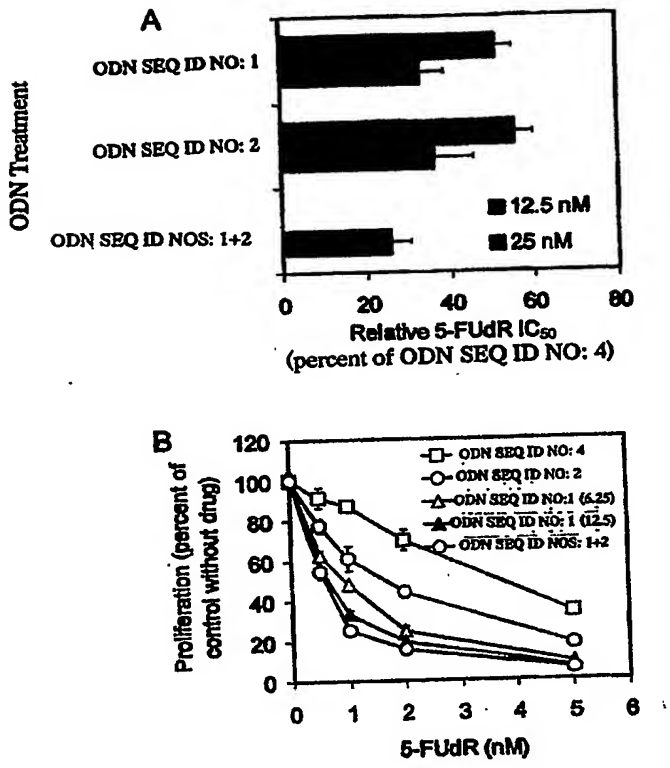
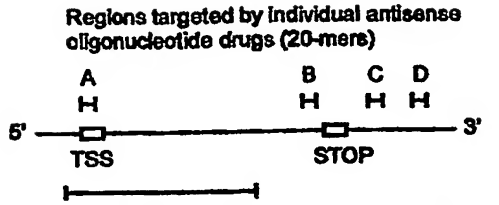

A
ODN SEQ ID NO: 1
ODN SEQ ID NO: 2
ODN SEQ ID NOS: 1+2
ODN Treatment
12.5 nM
25 nM
0
20
40
60
80
Relative 5-FUdR IC50
(percent of ODN SEQ ID NO: 4)
B
Proliferation (percent of control without drug)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
5-FUdR (nM)
ODN SEQ ID NO: 4
ODN SEQ ID NO: 2
ODN SEQ ID NO:1 (6.25)
ODN SEQ ID NO: 1 (12.5)
ODN SEQ ID NOS: 1+2

Figure 6



A: SEQ ID NO: 11 CATGCCGAATACCGACAGGG
B: SEQ ID NO: 2 TTGGATGCGGATTGTACCCT
C: SEQ ID NO: 1 GCCAGTGGCAACATCCTTAA
D: SEQ ID NO: 3 ACTCAGCTCCCTCAGATTTG

Isis 2nd generation ODNs

- 20-mers, fully phosphorothioated
- 6 bases on each end 2'-methoxyethoxylated
- Middle 8 bases unmodified
- Optimum RNase H activity

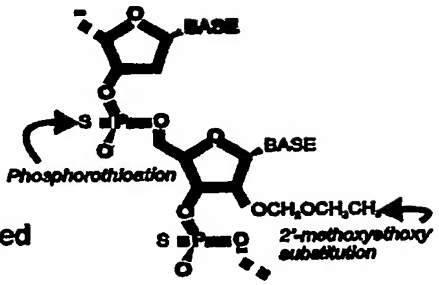


FIGURE 7